Polymorphisms of Platelet Glycoproteins in Relation to Macrovascular Disease in Type 2 Diabetes Mellitus

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We set out to determine the genotype distributions of the PI^A polymorphism of platelet glycoprotein IIIa, the HPA-3 polymorphism of platelet glycoprotein IIb, and the variable number tandem repeat (VNTR) polymorphism of platelet glycoprotein Ib in subjects with Type 2 diabetes mellitus (Type 2 DM) with ($n = 125$) and without ($n = 90$) a clinical history of macrovascular disease. In 215 white European subjects with Type 2 DM, presence of coronary artery disease was determined as a clinical history of angina, myocardial infarction (MI), coronary angioplasty or coronary artery by-pass grafting. Presence of peripheral vascular disease was defined as a clinical history of intermittent claudication with confirmatory vascular ultrasound or angiography, intermittent claudication with undetectable foot pulses and no history of arthralgia or surgery for leg ischaemia, confirmed by reference to medical case notes. Polymorphisms were detected by polymerase chain reaction amplification of DNA. There was no difference in the genotype distributions of subjects with and without macrovascular disease. In subjects with a first MI before the age of 60 years ($n = 26$), there was a 38 % incidence of PI^{A2} compared to 29 % in subjects free from clinically evident macrovascular disease, but this difference did not reach statistical significance. This study does not support the hypothesis that polymorphisms of platelet glycoproteins, in particular the PI^A polymorphism of platelet glycoprotein IIIa, play an important role in the pathogenesis of macrovascular disease in subjects with Type 2 DM. © **1998 John Wiley & Sons, Ltd.**

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Introduction

Type 2 diabetes mellitus (DM) is characterized by premature atherosclerosis and classical risk factors contribute to, but do not totally account for, the increased risk of atherosclerotic disease.[1]

Subjects with Type 2 DM have higher circulating levels of β-thromboglobulin and platelet factor 4, which have been used as markers of platelet activation.[2,3] In addition, platelets from diabetic subjects have been demonstrated to be hypersensitive to a variety of activators including ADP, thrombin, and collagen.[4] This hypersensitivity is related to a reduction in platelet membrane fluidity[5] and this has been associated with hyperglycaemia.[6] Subjects with hypercholesterolaemia also have hypersensitive platelets which bind increased amounts of fibrinogen and this, at least in part, may explain the platelet hypersensitivity observed in Type 2 DM.[7] It has also been observed that subjects with DM have increased circulating fibrinogen,[8] the major ligand for the platelet glycoprotein IIb/IIIa receptor involved in platelet aggregation,[9] and elevated fibrinogen levels have been associated with the development of coronary artery disease (CAD) in subjects with Type 2 DM and in non-diabetic subjects.[10–12] Thus, the interaction of fibrinogen with its platelet receptor may play an important role in the pathogenesis of thrombosis.

A number of polymorphisms of the platelet glycoproteins have been identified, including the PI^A polymorphism of platelet glycoprotein IIIa, the HPA-3 polymorphism of GPIIb and a variable number tandem repeat (VNTR) polymorphism of GPIbα.[13] PI^A has been associated with coronary thrombosis as assessed by the presence of acute myocardial infarction (MI) or unstable angina in a population with an approximately 20 % incidence of diabetes,[14] although this finding has not been confirmed by a number of other studies.[15] We have found a weaker association of this polymorphism with MI in subjects characterized by coronary angiography in a population

Abbreviations: CABG coronary artery bypass grafting, CAD coronary artery disease, IC intermittent claudication, MI myocardial infarction, PVD peripheral vascular disease, VNTR variable number tandem repeat

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with a low incidence of diabetes.[16] We therefore hypothesized[16] that this polymorphism may play a greater role in the development of vascular disease in subjects with diabetes.

The aim of this study was, therefore, to determine the association of these polymorphisms with the presence of macrovascular disease (either CAD or peripheral vascular disease (PVD)) in subjects with Type 2 diabetes mellitus.

Patients and Methods

Subjects

Two hundred and fifteen white European subjects with Type 2 diabetes mellitus, as defined by WHO criteria, were recruited from the diabetic clinic at the Leeds General Infirmary, as previously reported.[17] Each subject gave informed consent according to a protocol approved by the United Leeds Teaching Hospitals Research Ethics Committee. The presence of CAD was determined as a clinical history of angina; myocardial infarction (MI); coronary angioplasty or coronary artery by-pass grafting (CABG), confirmed by reference to medical case notes. Presence of peripheral vascular disease was defined as a clinical history of intermittent claudication (IC) with confirmatory vascular ultrasound or angiography; IC with no detectable foot pulses and no history of arthralgia, or surgery for leg ischaemia, confirmed by reference to medical case notes.

Analytical Methods

Venous blood samples were taken for haemostatic factors and blood lipids after an overnight fast and analysed as previously described.[18] The PI^A polymorphism was determined as previously reported.[19] Primers, designed to flank the VNTR region, were modified from those described by Simsek *et al*.:[20] forward 5′ CAC TAC TGA ACC AAC CCC AAG 3′ and reverse 5′ TTG TGG CAG ACA CCA GGA TGG 3′ to give total fragment lengths of 197 to 314 bp depending on the number of repeats. The HPA-3 polymorphism was detected by polymerase chain reaction (PCR) amplification using primers described by Unkelbach *et al*.[21] PCR conditions of 25 pmol each primer, 100 ng DNA, 200 μM each dNTP, 20 mM tris HCl (pH 8.4), $MgCl_2$ (1.5 mM for VNTR and 2.0 mM for HPA-3), 50 mM KCl, 0.05% W-1 and 0.75 u *Taq* DNA polymerase (GIBCOBRL) were used, involving 32 cycles of 93 °C for 1 min denaturing, 1 min annealing (at 67 °C for VNTR and 68 °C for HPA-3) and 1 min at 72 °C for extension followed by a final 5 min extension time at 72 °C. For determination of HPA-3 genotype, PCR products were restricted with 4 U *Fok* I restriction enzyme at 37 °C for 3 h. Genotypes were determined by 2 % agarose gel electrophoresis containing ethidium bromide visualized by ultraviolet light and sized with reference to a DNA ladder. VNTR polymorphism was classified according to the scheme of Moroi *et al*.[22] as D (single copy), C (2 copies), B (3 copies), and A (4 copies). HPA-3 polymorphism was classified as aa (Ile, Ile), ab (Ile, Ser), bb (Ser, Ser). PI^A was classified as A1/A1 (Leu, Leu), A1/A2 (Leu, Pro), and A2/A2 (Pro, Pro).

Statistical Analyses

The distributions of fibrinogen, BMI, triglycerides, and HbA_{1c} were positively skewed and data were therefore log transformed and analysed by parametric tests. Group data were compared by unpaired *t*-tests and results expressed as mean or geometric mean and (anti-logged) 95% confidence intervals. Duration since diagnosis of diabetes was compared between groups by Mann-Whitney U test and results expressed as median and interquartile range. Chi-squared analysis was used to determine differences in genotype frequencies and other categorical variables between subjects with and without macrovascular disease. Logistic regression models were used to identify independent predictors of CAD, PVD, and macrovascular disease. All statistical analyses were performed using the SPSS for Windows statistical package (SPSS Inc., Chicago, USA).

Results

Of the 215 subjects with DM, 71 had a clinical history of CAD (8 CABG, 2 coronary angioplasty, 47 MI, and 14 stable angina pectoris) and 69 of PVD (11 amputation, 10 reconstructive surgery or leg angioplasty, 5 proven stenoses, and 43 IC). The characteristics of DM subjects with and without clinical evidence of macrovascular disease are presented in Table 1. Subjects without macrovascular disease were significantly younger than those with macrovascular disease; however, there was no significant difference in duration of diabetes, BMI or HbA_{1c} levels between the two groups. Subjects with macrovascular disease had higher levels of cholesterol, triglycerides, and fibrinogen compared to those without.

In subjects with and without clinically evident macrovascular disease the genotype distributions of PI^A, HPA-3, and VNTR did not differ significantly from that predicted by Hardy-Weinberg equilibrium. The genotype distributions of subjects with and without macrovascular disease and with CAD and PVD are presented in Table 2. Genotypes were not available in 7 subjects for VNTR, 4 for PI^A, and 2 for HPA-3. There was no significant difference in the genotype distributions of the three polymorphisms in subjects without macrovascular disease compared to all subjects with macrovascular disease or those with either PVD or CAD. We determined the genotype distributions of subjects with MI before the age of 60 years ($n = 26$) compared to those free of macrovascular disease; 38 % ($n = 10$) of these subjects possessed the PI^{A2} allele compared to 29 % in healthy subjects, but again this did not reach statistical signifi-

Table 1. Characteristics of subjects with Type 2 diabetes mellitus with and without clinical evidence of macrovascular disease

	No macrovascular disease ($n = 90$)	Macrovascular disease ($n = 125$)	p value
Age (yr)	62.2 (59.9–64.4)	66.8 (65.1–68.5)	0.001
Cholesterol (mmol l^{-1})	5.8 (5.6–6.0)	6.2 (6.0–6.5)	0.02
Duration (yr)	5.3 (2.6–8.1)	6.1 (3.0–11.0)	ns
BMI (kg m^{-2})	28.8 (27.8–29.8)	28.0 (27.1–28.9)	ns
Fibrinogen (gl^{-1})	2.97 (2.82–3.12)	3.41 (3.18–3.65)	0.002
HbA_{1c} (%)	6.8 (6.5–7.1)	7.1 (6.9–7.3)	ns
Triglycerides (mmol l^{-1})	2.1 (1.9–2.3)	2.5 (2.2–2.8)	0.03
Sex (M:F)	48:42	72:53	ns
Ever smoked (no/yes)	53/37	76/49	ns
CAD	–	71	–
PVD		69	–
Hypertension (no/yes)	60/30	72/53	ns

Duration presented as median (interquartile range), other levels expressed as mean or geometric mean (95% confidence intervals).

Table 2. Genotype distributions of PI^A, HPA-3, and variable number tandem repeat (VNTR) polymorphisms in subjects with and without macrovascular disease and with coronary artery disease (CAD) and peripheral vascular disease (PVD)

		No macrovascular disease ($n = 90$)	Macrovascular disease ($n = 125$)	CAD ($n = 71$)	PVD ($n = 69$)
VNTR:	DD	2	4	2	2
	CD	8	7	4	4
	CC	74	110	64	59
	BC	2	–	–	–
	BB	1	–	–	–
HPA-3:	aa	34	57	33	32
	ab	47	56	32	30
	bb	8	11	5	5
PI^A:	A1/A1	64	87	47	46
	A1/A2	24	30	20	17
	A2/A2	2	4	–	4

No significant difference in genotype distributions at $p < 0.05$.

cance. There was no difference in levels of risk factors presented in Table 1 by genotype (data not shown).

In a stepwise logistic regression model comparing all subjects with macrovascular disease to subjects free of macrovascular disease, including age, duration of diabetes, hypertension, VNTR, PI^A, and HPA-3, only age was independently associated with the presence of macrovascular disease, with an odds ratio (95 % CI) for an increase of 10 years in age of 1.51 (1.47–1.56). Similarly, in models comparing subjects with CAD or PVD to those free of clinically evident macrovascular disease, age was the only factor independently associated with the presence of disease (data not shown).

Discussion

The VNTR polymorphism of GPIb results in one to four repeats of a 13 amino acid sequence in the macroglycopeptide region of the mature platelet glycoprotein.[23] It has been postulated that this might result in a variation in the length of the extracellular portion of the protein, leading to extension of the von Willebrand factor binding site, found at the amino terminal of this protein, further into the circulation with increasing number of motifs.[23] This raises the possibility that this VNTR may be causally associated with coronary thrombosis. The HPA-3 polymorphism of GPIIb is a common polymorphism in the extracellular region of the protein and has not been extensively studied in relation to vascular disease.[13] The PI^A polymorphism has been associated with MI, particularly in young subjects in whom we have found evidence of an interaction with cholesterol.[24] The strongest association of PI^{A2} was reported by Weiss *et al*. in subjects with MI or unstable angina in whom there was an almost 20 % incidence of diabetes.[14]

Type 2 DM is characterized by premature atherosclerosis and thrombosis. It has been demonstrated that

platelets from subjects with diabetes are hypersensitive to aggregating agents and this is thought to be related to both the hyperglycaemia and hyperinsulinaemia associated with this disorder.[25] Therefore it is possible that there may be an interaction of the platelet glycoprotein polymorphisms with the clustering of risk factors associated with the insulin resistance syndrome, enhancing the development of macrovascular disease in Type 2 diabetic subjects.

In the present study, as with other studies in Caucasian subjects,[20,23] we found no individuals possessing the A allele of the VNTR, despite its documented prevalence in Oriental subjects.[22,26] The genotype frequencies of PI^A and HPA-3 observed in this study were in keeping with previously reported frequencies.[21] We have found no difference in the genotype distributions of these three polymorphisms in subjects with macrovascular disease compared to those clinically free from vascular disease. Nor was there a difference in distributions in those with CAD or PVD individually when compared to those without macrovascular disease.

The association of PI^A with vascular disease has not been supported by a number of studies,[15] including the large prospective Physicians' Health Study[27] and this has lead to speculation about the functional relevance of this polymorphism.[28] Since atherosclerosis and thrombosis are complex disorders, any effect of PI^A may be influenced by other environmental and genetic factors; these factors will cluster differently in different populations and this may explain the inconsistent findings reported so far. The strongest associations have previously been reported in young subjects with acute thrombosis,[14,24] suggesting that age may be an important factor. The mean age of subjects in the present study was >60 years in both subjects with and without macrovascular disease. When we restricted our analyses to subjects with a first MI before the age of 60, we found 38 % of these subjects were PI^{A2} positive compared to 29 % in subjects without macrovascular disease, although this difference did not reach statistical significance. As Type 2 DM is characterized by early onset of macrovascular disease it is possible that PI^{A2} does play a role in these subjects but is related to even earlier events and possibly premature mortality which, along with the relatively small numbers of subjects with MI before the age of 60, may explain the lack of association observed in this study. In addition, there is a high incidence of silent ischaemia in subjects with Type 2 DM, which may have served to decrease the power of this study to detect a difference in genotype distributions.

The results of this study do not support our hypothesis[16] that polymorphisms of platelet glycoproteins, in particular the PI^A polymorphism of platelet glycoprotein IIIa, play an important role in the pathogenesis of macrovascular disease in subjects with Type 2 DM. Further larger studies will need to be carried out in order to determine whether there is an association of PI^{A2} with MI in young subjects with Type 2 DM.

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